REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	20 Apr 99	Final, 1 May 95 - 30 Apr 98

4. TITLE AND SUBTITLE
Exploiting Molecular Diversity of Enzymes Based on Phage Display: Development of Novel Enzymatic Catalysts

5. FUNDING NUMBERS
G N00014-95-10943

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8. PERFORMING ORGANIZATION REPORT NUMBER
Not applicable

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research, Code 1141SB
800 N. Quincy Street
Arlington, VA 22217

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
Not applicable

11. SUPPLEMENTARY NOTES
Distribution Unlimited

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Barnase was successfully displayed on the surface of m13 phage as an N-terminal fusion with coat protein III. The successful display was verified by Western Blotting of purified phage. For selection of a thermostable barnase, the buried residues Alall, Leu14, Leu20, Ala74, Ile88, Tyr90, and Ile96 were replaced randomly with Phe, Leu, Val, Ile, Ala, Pro, Gly, Ser, or Thr, giving a library of $2x10^6$ members. The transformation yield obtained ($4x10^6$) allows sampling of all possible members. We are currently selecting the phage library on immobilized barstar (the femtomolar inhibitor of barnase) at elevated temperature. Barstar N7C, a mutant constructed in this lab, was linked via a disulfide to a chromaography support. This allows specific elution under mild reducing conditions. Barnase has also been successfully expressed on the surface of phage when attached to the C-terminus of an Fab light chain, with the heavy chain attached to the gene III protein. Again, western blotting analysis was used to verify the success of display. Replacement of the barnase gene with cDNA should allow the successful surface display of cDNA libraries on phage.

14. SUBJECT TERMS
Barnase, Phage Display, Thermostability, DNA Library

15. NUMBER OF PAGES
5

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

FINAL REPORT

GRANT #: N00014-95-10943

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GRANT TITLE: Exploiting Molecular Diversity of Enzymes Based on Phage Display: Development of Novel Enzymatic Catalysts

REPORT PERIOD: May 1, 1995 - April 30, 1998

AWARD PERIOD: May 1, 1995 - April 30, 1998

OBJECTIVE: To engineer the stability, substrate specificity, and catalytic activity of enzymes using m13 phage display; to design a new and efficient system for selecting clones from cDNA libraries based on their binding properties.

APPROACH: The *Bacillus amyloliquefacians* ribonuclease, barnase, is used as a test protein due to its many favorable properties which include small size (110 amino acids), available crystal structure, ability to unfold and refold readily. For the selection of barnase mutants, phagemids are constructed in which the barnase gene is inserted between the m13 phage gene III ribosomal binding site/signal peptide coding sequence and the 3' end of gene III (residues 198-406). Barstar, an intracellular protein inhibitor of barnase, is also encoded by the phagemid, as this precludes the cytotoxicity of barnase. Following the verification that barnase is successfully displayed on the phage surface, mutagenesis of the barnase gene is performed by overlap extension PCR (polymerase chain reaction) using primers doped at specific locations with randomized codons. The locations for mutagenesis are selected based on structural information obtained from the crystal structure. For the selection of thermostable mutants, only buried (non-solvent accessible) residues are mutated, and these are mutated selectively to other hydrophobic residues in hopes of improving hydrophobic packing by replacing those codons with NYT (N = A+T+G+C, Y = T+C) rather than the

usual NNS (S = G+C). This strategy selectively replaces those residues with Phe, Leu, Ile, Val, Ser, Pro, Thr, or Gly and avoids all of the charged and many of the polar amino acids. This is an important simplification, because it greatly reduces the size of the library required (8 variants at each mutation site as opposed to 32 variants at each mutation site.)

Selection of thermostable barnase mutants is performed by selecting for phage that can bind to the femtomolar inhibitor, barstar, at elevated temperature. Wild type barnase melts at 50°C, while barstar melts at 70°C; this allows a 20°C window for selection.

For the more complex methodology of constructing cDNA libraries on the phage surface, an alternate strategy must be adopted. Because cDNA molecules have a stop codon at the 3' end, the genes cannot be placed at the 5' end of gene III. Display at the 3' end of gene III is not possible, however, since the C-terminus of coat protein III (encoded by the 3' end of gene III) is buried within the phage. Another coat protein used for phage display, coat protein VIII, suffers from the same problem. Recently, a third coat protein which has an exposed C-terminus has been used for phage display of a cDNA library, but it is unclear how large an insert can be effectively displayed by this system. We have proposed another strategy: barnase, again used as a test protein, is inserted *after* the gene for the light chain of an Fab (Fragment: antigen binding, the "business end" of an immunoglobulin), with the Fab heavy chain inserted between the signal peptide and the 3' end of the m13 phage gene III. This causes production of the barnase-light chain fusion as a soluble protein in the periplasmic space of *E. coli*, where it dimerizes with the heavy chain-coat protein III fusion. This dimerization may in fact be covalent, if the interchain disulfide bonds form properly. The sequestration of these chains in the periplasm prior to phage assembly and extrusion should prevent the soluble light chain from "shuffling" between the displayed heavy chains from different clones.

ACCOMPLISHMENTS. Barnase was successfully displayed on the surface of m13 phage using the construct containing the barnase gene attached to the 5' end of gene III. The successful display was verified by Western blotting of phage purified by polyethylene glycol precipitation followed by size exclusion chromatography on Sephacryl S-1000. The Sephacryl chromatography was included to remove

any soluble protein that might have been carried through the precipitation. The probe for the Western blot was a barstar-alkaline phosphatase fusion, made by reacting a barstar mutant containing a free thiol (barstar Asn7→Cys, made in this lab) with maleimide activated alkaline phosphatase. Asn7 is located at the opposite end of the protein from the barnase binding site, and derivatization at that position does not appear to interfere with barnase inhibition.

Several sites were selected for mutagenesis. For selection of a thermostable barnase, these were Ala11, Leu14, Leu20, Ala74, Ile88, Tyr90, and Ile96. Replacing the codons at these positions with NYT (N = A+T+G+C; Y = T+C) gives a library of $8^7 = 2x10^6$ members, which is easily achievable. Mutated barnase inserts were constructed by overlap extension PCR, and the mutants were ligated into the phage display vector in place of the wild type barnase. The number of transformants obtained following electroporation with the ligation mixture was assessed and found to be $4x10^6$, which should give adequate representation of the entire library. Barstar N7C was covalently linked to an activated glutathione-sepharose column. Because barstar binds to barnase with a very high affinity (femtomolar dissociation constant), an easily cleavable linker was necessary to allow gentle elution of bound phage from the support. By attaching barstar to the resin via a disulfide bond, elution can be accomplished under mild reducing conditions. As of the end of this grant period, we are beginning selection on this resin packed in a water-jacketed column. Selection will be performed at successively higher temperatures from 40°C up to 70°C. Negative controls have shown that nonspecific binding is quite minimal under these conditions.

Barnase has also been successfully expressed on the surface of phage when attached to the C-terminus of an Fab light chain, with the heavy chain attached to the gene III protein. Again, western blotting analysis was used to verify the success of display. Replacement of the barnase gene with cDNA should allow the successful surface display of cDNA libraries on phage.

CONCLUSIONS: Phage display is a potentially very powerful technique. The versatility of the method in terms of the types of proteins and protein assemblies that can be displayed is remarkable, and the number of clones that can be screened simultaneously makes this a potentially

powerful technology. Importantly, preliminary 40°C selection results indicate that under the conditions used (selection on a resin that displays little nonspecific binding, as opposed to a "sticky" microtiter plate; inclusion of "blocking" protein; using a specific elution technique as opposed to a non-selective elution technique such as high salt; and selection at a slightly elevated temperature), background nonspecific binding – which has been a thorn in the side of many phage display researchers – can be minimized.

SIGNIFICANCE: Currently, random mutagenesis still proves to be the best way to find enzymes of altered function or stability. Unfortunately, random mutagenesis often must be followed by a great deal of time-consuming screening. This work is part of an ongoing project to demonstrate the efficiency of phage display for selecting mutants of altered catalytic activity and thermal stability from large libraries. In addition, the demonstration that cDNA can be displayed on phage should open new pathways for rapidly cloning entire families of proteins based on their ability to bind certain ligands.

AWARD INFORMATION:

1995: ACS Melville L. Wolfrom Award in Carbohydrate Chemistry.

1995: Member of the NIH Bioorganic and Natural Products Chemistry Study Section

1996: Elected Member of The American Academy of Arts and Sciences

1998: ACS Harrison-Howe Award in Chemistry

PUBLICATIONS AND ABSTRACTS:

1. Sears, P. (1996) Use of Site-Directed Mutagenesis and Phage Display to Engineer Enzymes for Organic Synthesis. Ph.D. Thesis, The Scripps Research Institute.

2. Sears, P.; Wong, C.-H. (1996) Engineering Enzymes for Bioorganic Synthesis: Peptide Bond Formation. Biotechnol. Prog. 12: 423-433.